Further research on the production, longevity and infectivity of the zoospores of *Leptolegnia chapmanii* Seymour (Oomycota: Peronosporomycetes)

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ABSTRACT

The effect of temperature on the production, survival and infectivity of zoospores of an Argentinean isolate of *Leptolegnia chapmanii* was determined under laboratory conditions. Production of zoospores of *L. chapmanii in vitro* and *in vivo* upon first and fourth instars larvae of the mosquito *Aedes aegypti* was studied at three different temperatures. Zoospores from infected larvae were infective to mosquito larvae for 51, 12, and 5 consecutive days when maintained at 25, 35, and 10 °C, respectively. Maximum zoospore production in infected fourth-instar larvae was $9.6 \pm 1.4 \times 10^4$ zoosp/larva at 48 h at 25 °C. The average number of zoospores produced by individual fourth-instar *Ae. aegypti* larvae infected with *L. chapmanii* was $3.57 \pm 0.46 \times 10^5$ zoospores during 6 consecutive days at 25 °C. Zoospore production *in vitro* was also affected by temperature with a maximum of zoospores ($n = 47,666$/ml) produced at 25 °C. When zoospores produced *in vitro* were used as inoculum against *Ae. aegypti* larvae at 25 °C, larval mortality was recorded for 5 consecutive weeks. The encystment process for zoospores took 17–20 min; the germination of cysts (excystment) occurred 5 min after exposure in water to mosquito larvae. The minimal time of contact between zoospores and mosquito larvae to develop infection was two minutes. Infection took place by zoospore attachment onto and then penetration through the larval cuticle or by ingestion of cysts as was confirmed by histological studies. Temperature directly affected infectivity and production of zoospores *in vivo* and *in vitro* although *L. chapmanii* zoospores tolerate a wide range of temperatures.

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1. Introduction

Although a number of oomycete watermolds are parasitic on plants and animals, only *Lagenidium giganteum* has been widely recognized to have a significant impact as a natural control agent for mosquitoes (Umphlett and Huang, 1972; World Health Organization, 1979). Seymour (1976) reported the isolation of a watermold from a parasitized mosquito larva that he tentatively identified as a *Leptolegnia* species but he attempted no further infection experiments. In 1975, larvae collected in South Carolina, USA, were infected with a similar fungus,[2] even though not identical to the *Leptolegnia* reported by Seymour (Muehleisen, 1977). *Leptolegnia chapmanii* Seymour (1984) was isolated from several mosquito species (McInnis and Zattau, 1982; Seymour, 1984; Lord and Fukuda, 1988; Fukuda et al., 1997). López Lastra et al. (1999) iso-

lated *L. chapmanii* from the neotropical mosquito species *Ochlerotatus albifasciatus* (Macquart) in Argentina, this was the first report of this pathogen from the Southern Hemisphere. López Lastra et al. (2004) determined the susceptibility of 10 species belonging to 5 genera of mosquitoes to the native isolate of *L. chapmanii* under laboratory conditions. Natural breeding sites for these mosquito species are characterized by a wide variety of biotic and abiotic conditions. A wide range of tolerance of zoospores of the Argentinean isolate of *L. chapmanii* to biotic and abiotic factors such as temperature, pH, and NaCl concentration was determined under laboratory conditions (Pelizza et al., 2007a,b). Additional research conducted in this study examined production, survival and infectivity of zoospores of *L. chapmanii* to better establish parameters that affect survival and epizootics under natural conditions.

Specifically, laboratory studies were conducted to determine the effect of temperature on zoospore production in artificial media and in infected mosquito larvae at three different temperatures. In addition, survivorship of zoospores measured through infectivity in mosquito larvae was determined at 10, 25, and 35 °C. The times needed for encystment and excystment of zoospores were determined as was the minimal time of contact with zoospores for mosquito larval infection to occur.

2. Materials and methods

2.1. Fungal culturing

The Argentinean isolate of *L. chapmanii* (CEP 010, ARSEF 5499) was maintained on Emerson's YpSS agar media (yeast extract 4 g, HK_2PO_4 1 g, $MgSO_4$ 0.5 g, starch 15 g, agar 20 g, distilled water 1000 ml) in 60×15 mm sterile Petri dishes. Inoculum was prepared by cutting 5 mm cubes of agar, and mycelial blocks containing hyphae were placed in 20 ml of sterile distilled water in 90×15 mm sterile Petri dishes and incubated at 25 ± 0.5 °C for 3 days. When zoospores were observed in the water, fourth-instar *Ae. aegypti* were placed in these Petri dishes. Dead larvae were removed and examined with phase contrast microscopy to confirm fungal infection. *L. chapmanii*-infected *Ae. aegypti* larvae, 48 h post-infection (p.i.), were used as fungal inoculum.

2.2. Mosquito larvae

The larvae of *Ae. aegypti* used in this study were obtained from a colony maintained at the Centro de Estudios Parasitológicos y de Vectores (CEPAVE, La Plata, Argentina) following standard mosquito breeding techniques (Gerberg et al., 1994).

2.3. Production (total and daily) of zoospores of L. chapmanii in infected Ae. aegypti larvae

One thousand first- and fourth-instar *Ae. aegypti* larvae were placed in each of six plastic containers (three containers for each instar) with 2000 ml of distilled water and maintained at 10, 25, and 35 °C. Ten *L. chapmanii*-infected fourth-instar larvae 48 h p.i. were added to each of the containers with healthy larvae. Ten exposed larvae were removed by forceps from the container every hour from time 0 to 24 h post-exposure. After 24 h, 25 larvae were removed from the container every 24 h until no further zoospores were present on the larva cuticle Exposed larvae were individually macerated in an Eppendorf tube with 0.5 ml of phosphate-buffered saline and fixed in 0.5 ml of 2.5% glutaraldehyde. Mature and incompletely differentiated zoospore numbers at a given time point were determined in a hemocytometer (Neubauer chamber) at $40\times$ magnification with an Olympus BX41 phase contrast compound microscope. The number of (mature) swimming zoospores, recognized by their shape and the positions of their flagella, and of incompletely differentiated zoospores presented on the larva surface every 24 h in infected mosquito larvae were determined under laboratory conditions.

The total number of zoospores released daily for a single *L. chapmanii* infected mosquito larvae was also determined. Fourth-instar *Ae. aegypti* larvae ($n = 6$) after 1 h exposed to the zoospore suspension of *L. chapmanii* were individually placed with forceps in 1 ml distilled water in each well of 24-multiwell tissue culture plates, and maintained at 25 °C. Every 24 h each infected larva was moved to a new well with 1 ml of water until no new zoospores were detected. The number of swimming and encysted zoospores presented in the previous well was determined in a Neubauer chamber. Experiments described above were replicated three times on different dates under similar laboratory conditions.

2.4. Time of encystment of motile zoospores

For a better understanding of the dynamics of zoospores released by an infected larva, the period for actively searching and time for spore encystment were determined in a laboratory experiment. One milliliter (approximately 1000 zoospores) of a fresh zoospore suspension was placed in a depression slide under a phase contrast microscope, and the time needed for zoospore encystment was determined by continuous observation of the zoospores at $40\times$ magnification. This experiment was replicated twice on different dates under similar conditions.

2.5. Time effect on the longevity and infectivity of L. chapmanii zoospores at 10, 25, and 35 °C

Leptolegnia chapmanii-infected fourth-instar *Ae. aegypti* larvae 48 h p.i. were individually placed in 24-multiwell cell culture plates (11 plates) and with 2 ml of distilled water per well. One healthy fourth-instar *Ae. aegypti* larvae was added per well ($n = 264$; 198 treated + 66 controls) every 24 h. Plates were maintained at 10, 25, or 35 °C and 12:12 (L:D) photoperiod. Larval mortality was recorded daily, and dead larvae were examined for fungal infection in wet mount preparations with phase contrast microscopy.

In addition, thirty 60-mm Petri dishes (3 per week over 10 weeks) with 10 ml of distilled water and a zoospores suspension (94,000 zoosp.) obtained from *in vitro* cultures in Emerson's YpSS agar media were maintained at 10, 25, and 35 °C during the 10 weeks study. Every week, 10 early fourth-instar larvae were added to each of the three dishes. *L. chapmanii* infection was recorded in exposed larvae under a light microscope at 24, 48, 72, and 96 h. The experiments described above were replicated three times on different dates under similar laboratory conditions.

2.6. Effect of the temperature on production of zoospores in vitro

Leptolegnia chapmanii was maintained on Emerson's YpSS agar media in 60×15 mm sterilized Petri dishes. Inoculum was prepared by placing cubes of agar (0.5 cm each dimension) containing hyphae in 20 ml of sterile distilled water in sterile 90-mm Petri dishes subsequently incubated at 10, 25, and 35 ± 0.3 °C. At 72 h, zoospore production in each Petri dish at these three temperatures was quantified with a hemocytometer. This experiment was replicated three times on different dates under similar conditions.

Infectivity of the zoospores produced *in vitro* at 10, 25 and 35 °C was determined by exposing 25 healthy fourth-instar *Ae. aegypti* larvae to a zoospores suspension in containers with 100 ml of water maintained at 25 °C. Three replicates and one control without zoospores and three repetitions on different dates were conducted for this experiment.

2.7. Time for zoospore excystment

A suspension of zoospores ($n = 94,000$ zoosp.) obtained from infected larvae was placed in Petri dishes with 10 ml of distilled water and maintained at 25 °C until all zoospores encysted. Groups of six fourth-instar *Ae. aegypti* larvae were placed in contact with the zoospore suspension for 1, 2, 3, 4, 5, 10, 20, 30, 40, 50, and 60 min, respectively. The groups of larvae were placed separately in containers with 100 ml of distilled water for 72 h, and examined microscopically for *L. chapmanii* infection. In addition, three samples of the cyst suspension were taken after each time point when larvae were removed and observed for motile zoospores (excysted zoospores) on wet mount preparations by light microscopy. This experiment was replicated three times on different dates under similar laboratory conditions.

2.8. Minimal exposure time for zoospore infection

Groups of 25 *Ae. aegypti* fourth-instar larvae were exposed to suspensions of 2×10^5 zoospores (1.4×10^5 cysts + 0.6×10^5 swim zoosp) for variable periods of time from 1 to 10 min in containers with 100 ml of water at 25 °C. After zoospore exposures, larvae were washed five times in containers with 100 ml of distilled

water and maintained individually in containers with 50 ml of distilled water at 25 °C. Mortality of larvae was recorded after 48 h, and wet mount preparations of all dead larvae were examined microscopically to confirm fungal infection. This experiment was replicated twice under similar laboratory conditions.

2.9. Infection route

A series of laboratory experiments were conducted to determine if mosquito larvae can become infected by ingestion of encysted zoospores in addition to the more routine penetration through the cuticle from encysted zoospores, and to investigate the relative importance of the two modes of entry of *L. chapmanii* in the overall infection rates of mosquito larvae. Third- or early fourth-instar larvae ($n = 200$) were exposed for 2 min to a suspension of encysted zoospores suspension ($n = 94,000$). Half of the exposed larvae were surface sterilized by 1 min of washing in 0.5% sodium hypochlorite solution (enough to kill newly attached zoospores but not toxic for the exposed larvae), and then placed in a container with 100 ml of dechlorinated water for 24 h. The other half of the exposed larvae were also placed in similar conditions without surface sterilization. After this period, three of the exposed larvae were fixed in Bouin's solution every 60 min for 24 h using routine histological methods, embedded in paraffin and stained with hematoxylin and eosin (Luna, 1968) or Grocott methenamine silver stain contrasted with light green (Ferron et al., 1966; Dikgolz et al., 2005) in order to investigate presence and/or germination of *L. chapmanii* cysts, midgut tissue and hemocoel invasion. Sterilized and not sterilized larvae not processed for histological studies maintained at 25 °C for 24 h were observed to determine *L. chapmanii* overall infection in both groups. This experiment was replicated three times on different dates under similar laboratory conditions.

3. Results

3.1. Production of L. chapmanii zoospores in infected Ae. aegypti larvae

The presence of zoospores on larval surfaces was detected as early as 14 h p.i. with an average of 150 ± 92 zoospores per fourth-instar larva at 25 °C (Fig. 1); of these zoospores, 37 ± 17 were mature and the rest incompletely differentiated. This zoospore number increased rapidly to a maximum of $9.6 \pm 1.4 \times 10^4$ zoosp/larva (61,000 incompletely differentiated and 35,000 mature zoospores) at 48 h and decreased to approximately 9800 zoosp/larva (7100 incompletely differentiated and 2700 mature zoospores) by day 6 p.i. (Fig. 1). Infected larvae incubated at 10 °C, initiated production of zoospores at 22 h p.i. when $1.7 \pm 0.9 \times 10^3$ zoosp/larva (310 mature zoosp) were produced. Zoospore production increased to as many as $1.69 \pm 0.23 \times 10^4$ zoospores/larva (5434

mature zoosp) at 48 h, and then decreased to $2.9 \pm 0.9 \times 10^3$ zoosp/larva (1200 mature zoosp) at 120 h p.i., no zoospores were produced after this time (Fig. 1). At 35 °C, low zoospore production was recorded at 22 h ($3.6 \pm 0.84 \times 10^3$ zoosp/larva with 1350 mature); the highest presence of zoospores on larval surfaces at a given time point was $1.85 \pm 0.16 \times 10^4$ zoosp/larva at 48 h (5000 mature zoosp) and decreased to $4.9 \pm 0.9 \times 10^3$ zoosp/larva (2840 mature zoosp) at 120 h (Fig. 1).

First-instar larvae of *Ae. aegypti* infected with *L. chapmanii* at 25 °C initiated zoospore production as early as 16 h p.i. producing an average of 121 ± 40 zoosp/larva (87 incompletely differentiated zoospore + 34 swim zoosp.) (Fig. 2). Zoospore production increased until 48 h p.i. with $5.5 \pm 0.3 \times 10^4$ zoosp/larva (20,326 mature zoosp) and decreased to $1.8 \pm 0.2 \times 10^4$ zoosp/larva (8800 swim zoosp) at 96 h (Fig. 2). Zoospore production in infected first-instar larvae maintained at 10 °C was initiated at 22 h p.i. (683 ± 216 zoosp/larva), with the highest number of zoospores recorded at 48 h with $5.02 \pm 0.8 \times 10^3$ zoosp/larva (2,480 swim zoosp) and a minimum of 884 ± 249 zoosp/larva (435 swim zoosp) at 96 h (Fig. 2). At 35 °C, zoospore production in infected first-instar larvae started at 22 h p.i. ($1,090 \pm 352$ zoosp/larva, with 270 swim zoosp), reached the highest production at 48 h with an average of $5.3 \pm 1.1 \times 10^3$ zoosp/larva (2934 mature zoosp), and decreased after 96 h with 1757 ± 723 zoosp/larva (940 swim zoosp) (Fig. 2). All first-instar larvae disintegrated completely after 100 h p.i. due to *L. chapmanii* infections, and the experiment was discontinued.

The overall total average zoospores produced by individual fourth-instar *Ae. aegypti* larvae infected with *L. chapmanii* was $3.57 \pm 0.46 \times 10^5$ zoospores over 6 days at 25 °C.

3.2. Production and infectivity of zoospores produced in vitro at different temperatures

An average of $7.58 \pm 0.36 \times 10^4$ zoosp/ml (47,800 cysts + 28,000 swimming zoospores) were produced for each 0.5 cm³ piece of mycelial culture placed in water for 72 h at 25 °C. Zoospore production decreased to $1.27 \pm 0.15 \times 10^4$ zoosp/ml (9700 cysts and 3000 swim zoosp) when mycelium was maintained 72 h in water at 35 °C and to $0.85 \pm 0.13 \times 10^4$ zoosp/ml (6500 cysts and 2000 swim zoosp) at 10 °C. Zoospores produced *in vitro* at those three temperatures were infective to *Ae. aegypti* larvae and caused 100% infection after 48 h.

3.3. Time effect on the longevity and infectivity of L. chapmanii zoospores at different temperatures

Zoospores were infective for 51 days at 25 °C and produced 100% larval mortality at 24 h throughout the first 25 days but de-
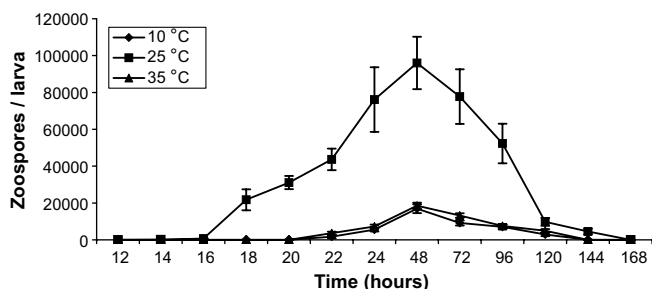

Fig. 1. Number (mean ± SD) of zoospores of *Leptolegnia chapmanii* on the surface of an individual fourth-instar *Aedes aegypti* larva at a given time point at three different temperatures.
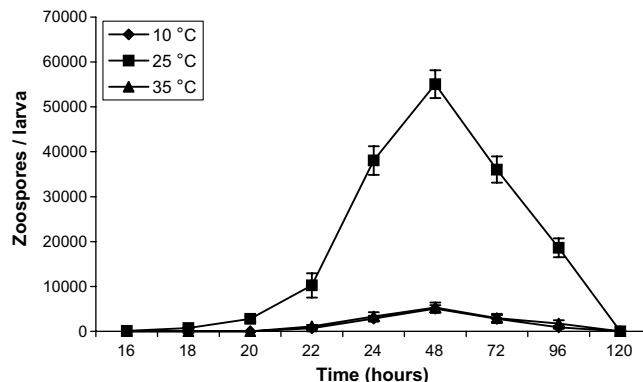

Fig. 2. Number (mean ± SD) of zoospores of *Leptolegnia chapmanii* on the surface of an individual first-instar *Aedes aegypti* larva at a given time point at three different temperatures.

creased to 10% mortality at day 51 (Fig. 3). After 51 days no further mortality was recorded in exposed larvae. Larval mortality rates were 100% at 24 h during the first 5 consecutive days when zoospores were maintained at 10 °C although after this time no mortality of exposed larvae was recorded (Fig. 3). When zoospores were maintained at 35 °C, 100% larvae mortality was recorded in 24 h for the first 12 days after which no further mortality was recorded (Fig. 3).

When zoospores produced *in vitro* were used as inoculum against *Ae. aegypti* larvae at 25 °C, larvae mortality was observed for 5 consecutive weeks (Fig. 4), with 100% mortality recorded at 24 h post-exposure for the first 2 weeks, at 48 h with the week 3 zoospores, at 72 h with the week 4 zoospores, and after 96 h with the week 5 zoospores. No larval mortality was recorded from 1 to 10 weeks among larvae exposed to a suspension of zoospores produced *in vitro* at 10 or 35 °C.

3.4. Zoospores encystment and excystment

Continuous light microscopic observation of free-swimming zoospores showed that these cells needed 17–20 min to settle onto the substrate, to retract their flagella, and then to encyst. Newly released zoospores from *L. chapmanii* sporangia were pyriform to drop-shaped and anteriorly biflagellate (typical primary zoospores); these zoospores swam very actively in roughly circular movements in all directions during the first 10 min. In the next 7–10 min the circular movements became less active until they only rotated around their own axis. Finally, the zoospores stopped moving, and rounded up, during which time the flagella also disap-
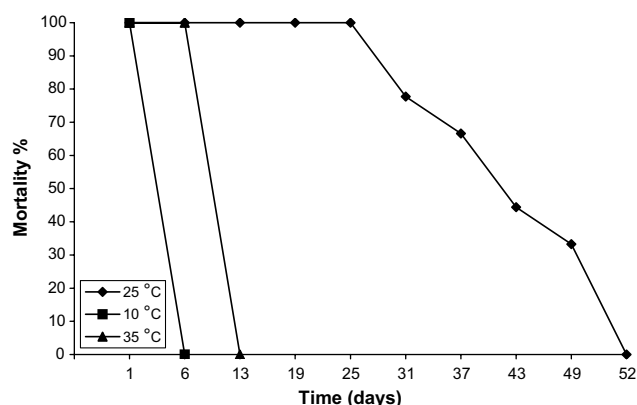

Fig. 3. Survival and infectivity of zoospores of *Leptolegnia chapmanii* from infected larvae on *Aedes aegypti* larvae at three different temperatures.
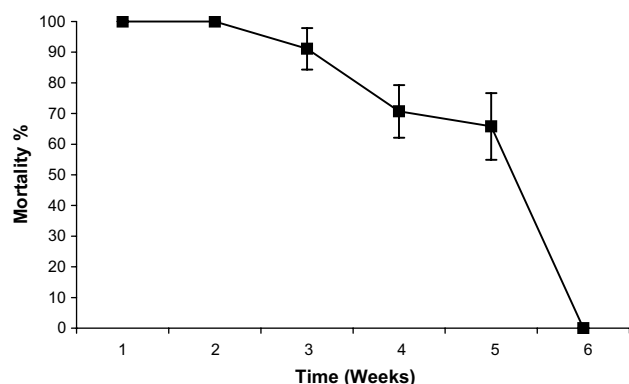

Fig. 4. Survival and infectivity of zoospores of *Leptolegnia chapmanii* produced *in vitro* in *Aedes aegypti* larvae at 25 °C.

peared (by resorption into the cell). Usually within 5 min of adding *Ae. aegypti* larvae to dishes containing encysted zoospores, the cyst walls were apparently digested, the cells began slow oscillatory movements and changed shape from spherical to kidney- or bean-like shape, and converted to laterally biflagellate (secondary) zoospores. These secondary zoospores then began very active movements, characteristic of *L. chapmanii* zoospores.

3.5. Minimal exposure time for zoospore infection

No larvae became infected when exposed for one minute to a suspension of 2×10^5 zoospores (1.4×10^5 cysts + 0.6×10^5 swim zoosp) but 100% infection resulted after larvae were exposed for two minutes or longer to the same *L. chapmanii* zoospore suspension. Phase contrast microscopic observations of wet mounts of larvae showed the presence of flagellate zoospores attached to the cuticle after as little as 2 min.

3.6. Per os infection by Leptolegnia and penetration through the larval gut

Cysts in the larvae gut were observed in surface sterilized larvae in wet mount preparations observed under a light microscope with phase contrast and in histological preparations. Germinated and ungerminated cysts were detected in the midgut lumen of surface-sterilized mosquito larvae as quickly as 5 min after the first exposure to the fungus (Fig. 5). Within 5–7 h of initial exposure to the fungus, vegetative growth of *L. chapmanii* was observable between the gut epithelium and the peritrophic membrane (Fig. 5). Hyphae traversing the gut epithelial cells and growing in the hemocoel were observed from 7 to 24 h p.i. (Fig. 5). The presence of the fungus there was confirmed by both phase contrast observations of wet mounts as well as by histological preparations of fixed and embedded larvae. Mortality rates of surface sterilized larvae exposed to a zoospore/cyst suspension for only two minutes (during which time fungus could be ingested) was $15 \pm 1.2\%$ and $35 \pm 2.3\%$ after 24 and 48 h, respectively, whereas larvae exposed to the identical inoculum but not then surface sterilized after 2 min showed fungus-induced mortalities of $40 \pm 0.9\%$ – $75 \pm 1.5\%$. Mortality of surface sterilized larvae not exposed to *L. chapmanii* zoospores suspension was not recorded.

4. Discussion

The life cycle of *L. chapmanii* is typical of saprolegnialean fungi (Zattau and McInnis, 1987). The asexual propagules (zoospores) are the infective units. Larval infection occurred by two methods, either by encystment of motile zoospores on the larval cuticle or by germination of ingested zoospore cysts in the host gut (Zattau and McInnis, 1987; Lord and Fukuda, 1988). As was demonstrated in this study only a very short time was required for *L. chapmanii* zoospores to find, and attach to the cuticle, and/or to be ingested by *Ae. aegypti* larvae. Germination of cysts and the vegetative growth recorded in the midgut of sterilized larvae constituted clear evidence of per os transmission of *L. chapmanii* as described by Zattau and McInnis (1987) and Lord and Fukuda (1988). The appreciable but lower infection rate among surface sterilized larvae after two minutes of contact with motile zoospores in which the only plausible route for infection was by ingestion of fungal units with subsequent infection through the gut clearly supported the ingestion of cysts as a way of entry for *L. chapmanii*. Although the lower rates of infection through the gut than through the cuticle suggest that infection through the gut is a comparatively inefficient mode of infection for this pathogen. The fact that *L. chapmanii* was capable of infecting larvae by two different modes enhances its ability to persist under natural conditions.
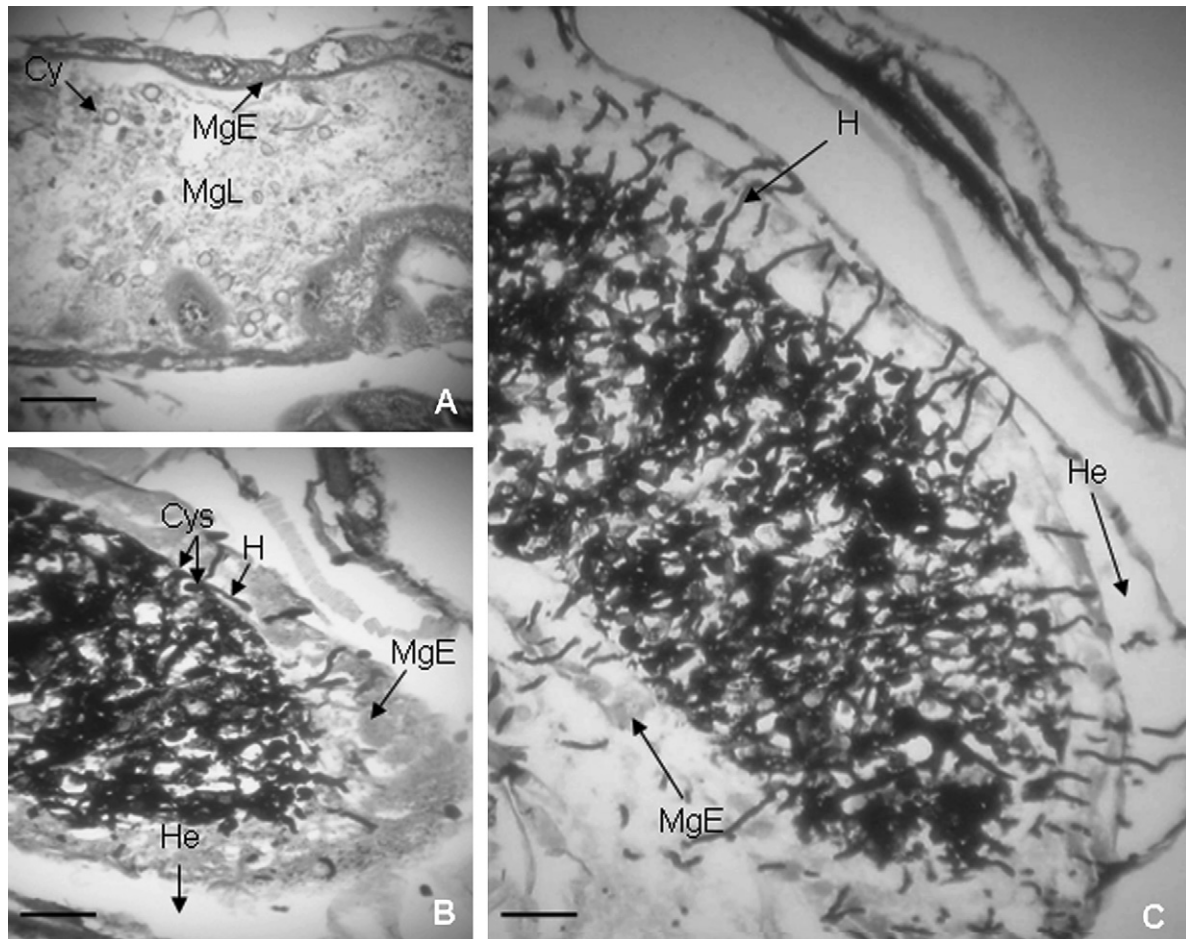
Fig. 5. Paraffin section of a surface sterilized *Aedes aegypti* larvae exposed for two minutes to a *Leptolegnia chapmanii* cysts suspension. (A) Presence of cysts (Cy) in the larval midgut lumen (MgL) at one hour post-ingestion. (Hematoxylin–Eosin stain). Bar = 50 μm. (B) *L. chapmanii* hyphae (H) traversing the gut epithelial cells (MgE) at 6 h post-ingestion (Grocott stain). Bar = 50 μm (C) *L. chapmanii* hyphae (H) growing in the hemocoel (He) at 9 h post-ingestion (Grocott stain). Bar = 25 μm.

Zoospore production was heavily affected by relatively low and high temperatures. Production was initiated as early as 14 h post-infection at 25 °C, and it continued throughout six consecutive days although most zoospores were produced 24–72 h post-infection. Reduced longevity and infectivity of zoospores produced *in vitro* was determined in this study in comparison with zoospores from infected mosquito larvae. Under laboratory conditions zoospores survived for more than 50 days as demonstrated by the ability of zoospores released from an infected fourth-instar larva to infect exposed mosquito larvae. These zoospores produced 100% larval mortality for almost a month. When zoospores produced *in vitro* were used as inoculum, larval mortality was recorded over 5 weeks with 100% mortality after 24 h incubation only during the first 2 weeks. *Lagenidium giganteum* Couch, an aquatic mosquito pathogen closely related to *L. chapmanii*, lost the ability to produce zoospores and oospores and to infect mosquito larvae after prolonged culture on sterol-free media (Lord and Roberts, 1986; Scholte et al., 2004) but such a loss of developmental capacity has not been corroborated here for *L. chapmanii*.

The zoospores are the usual infective units of this fungus against its mosquito hosts but this study demonstrated that these delicate motile spores cannot be stored for long periods by any routine method that allows the preservation of most other filamentous fungi. Any serious consideration to use *L. chapmanii* as a mosquito larvicide must still await the demonstration of means to produce, to store, to transport, and (in some appropriate form) to deliver large quantities of infective propagules to target mosquitoes.

Leptolegnia chapmanii has been isolated from a variety of larval mosquito species found in distinctive habitats including tree holes, artificial containers, freshwater/brackish flood planes, woodland ponds etc (Fukuda et al., 1997; López Lastra et al., 2004, 1999; Scholte et al., 2004). However, there is a lack of empirical data on factors that control the distribution and epizootics of this widespread natural control agent of mosquitoes. Abiotic factors that have been shown to affect the infectivity of zoospores of *L. chapmanii* are pH, temperature and salinity (Lord et al., 1988; Pelizza et al., 2007a,b). In this study, temperature was further evaluated to better define the effect on production, survival and infectivity of zoospores for this Argentinean isolate of *L. chapmanii*. This information contributes to a better understanding of the factors that enhance or limit the successful establishment and spread of *L. chapmanii* in different habitats and mosquito species.

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